CRISPR/Cas9-Mediated Deletionof *Foxn1* in NOD/SCID/IL2rg$^{-/-}$ Mice Results in Severe Immunodeficiency

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Abstract

Immunodeficient mice engrafted with either normal or cancerous human cells are widely used in basic and translational research. In particular, NOD/SCID/IL2rg$^{-/-}$ mice can support the growth of various types of human cancer cells. However, the hairs of these mice interfere with the observation and imaging of engrafted tissues. Therefore, novel hairless strains exhibiting comparable immunodeficiency would be beneficial. Recently, the CRISPR/Cas9 system has been used for efficient multiplexed genome editing. In the present study, we generated a novel strain of nude NOD/SCID/IL2rg$^{-/-}$ (NSIN) mice by knocking out *Foxn1* using the CRISPR/Cas9 system from NOD/SCID/IL2rg$^{-/-}$ (NSI) mice. NSIN mice were deficient in B, T, and NK cells, which showed impaired T cell reconstitution and thymus regeneration after allogeneic bone marrow nucleated cell transplantation, and exhibited improved capacities of grafting both leukemic and solid tumor cells compared with NSI, NOG and NDG mice. Moreover, NSIN mice facilitated the monitoring and in vivo imaging of both leukemia and solid tumors. Therefore, our NSIN mice provide a new platform for xenograft mouse models in basic and translational research.

Introduction

The development of immunodeficient mice engrafted with human cells or tissues ("humanized" mice), contributed significantly to translational biomedical research [1-3]. The discovery of athymic nude mice[4], which were first reported in 1966 as a spontaneously occurring phenotype, enabled the modeling of human tumors in immunodeficient mice[5]. Subsequent improvements include the severe combined immune deficient (SCID) [6] mutation, targeted mutations in recombination-activating genes 1 and 2 ($Rag1^{-/-}$ and $Rag2^{-/-}$)[7,8] that severely cripple the adaptive immune response of the murine host, and a mutation in the gene encoding the common γ chain of the interleukin 2 (IL2) receptor (IL2rg). NOD/SCID background with IL2rg mutations, such as NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl} (NSG)[9] and NODShi.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug}(NOG) mice[10], are able to grow almost all types of human cancers in vivo, including most solid tumors and hematological malignancies, and can be engrafted with functional human immune cells[11-14]. In our previous work, we generated a strain of NOD/SCID/IL2rg$^{-/-}$ (NSI) mice[15-19]. However, the hairs present on these strains impair the observation and imaging of engrafted tumors, necessitating the generation of nude NOD/SCID/IL2rg$^{-/-}$ mice. The nude [4]gene *Foxn1* (forkhead box N1) encodes a transcription factor for forkhead family proteins[20,21]. *Foxn1* is continuously expressed in the thymus, and is necessary for initial thymus organogenesis and the maintenance of cortical and medullary thymic epithelial cells (cTECs and mTECs) [22]in both embryonic[23] and postnatal mice[24-26]. Mutations in *Foxn1* cause inborn dysgenesis of the thymus and hairless skin [27].

A variety of methods have been developed for genome modification, including designer zinc fingers (ZFs), transcription activator-like effectors (TALEs), and the type II bacterial CRISPR/Cas9 system. Recently, the CRISPR/Cas9 system has been shown to be suitable for multiplexed genome editing[28,29]. The ease of design, construction, and delivery of multiple sgRNAs by co-microinjection[30-32] of Cas9 mRNA suggest that this system can be used to generate a variety of novel immunodeficient mouse strains.

In the present study, we derived a *Foxn1*mutated NOD/SCID/IL2rg$^{-/-}$ mouse (NSIN) strain using the CRISPR/Cas9 system. Cas9 mRNA and gRNA targeting *Foxn1* were injected into the cytoplasm of pronuclear-stage NSI mouse embryos. The mutant offspring were mated to generate homozygous NSIN mice. The NSIN mice were hairless and deficient in B, T, and NK cells, exhibited enhanced engrafting capacities for both leukemia and solid tumors compared with NSI and NOG mice. Moreover, the hairlessness facilitated the observation and imaging of tumors. Our study shows that NSIN mice can be used to generate ideal models for basic and translational research.

Results

Efficient modification of *Foxn1* in PL08 cells in vitro using CRISPR/Cas9

First, to test the targeting accuracy and efficiency of our CRISPR/Cas9 system, we designed gRNA targeting the first exon [33]of murine *Foxn1* (Figure 1A) and transfected plasmids expressing mammalian-codon optimized Cas9 and gRNA into a murine PL08 cell line [16] (Figure 1B). 24 h later, transfected cells were selected with 72 hours treatment of500 μg/ml of G418 and cell clones were picked.DNA was extracted from twenty cell clones to determine their genotypes in each experiment. DNA sequencing revealed cell clones that carried the expected mutation at the target locus (Figure 1C). The knock-out efficiency of *Foxn1* in PL08 cells was about 20% (Figure 1D). These data demonstrated the specific and efficient targeting of *Foxn1* by our CRISPR/Cas9 system.

Generation of NSIN mice by deletion of *Foxn1*

Then, with the CRISPR/Cas9 system with multiplexable genome engineering capabilities, we attempted to knock out the *Foxn1* gene from the NOD/SCID/IL2rg$^{-/-}$ background (NSI mice). *Foxn1* deleted mice were generated through direct embryo manipulation (Figure 2A). After in vitro transcription, a mixture of Cas9 mRNA (20 ng/μl) and gRNA for *Foxn1* (20 ng/μl) was microinjected into the cytoplasm of

pronuclear-stage embryos of NSI mice[28]. Blastocysts derived from the injected embryos were transplanted into foster mothers and 14 newborn pups were obtained (Table 1). Genomic DNA was extracted from the pups for PCR amplification. DNA sequencing revealed that one mouse carried the expected mutation at the target locus (Figure 2B). A new AluI restriction enzyme recognition site was generated by deleting a thymidine (Figure 2B). Then, a 123 bp fragment spanning the target site was amplified using PCR. The PCR products were digested with the AluI enzyme. The AluI digestion of the PCR products of wild-type, heterozygous and homozygous offspring generated fragments with lengths of 123 bp, 123+98+25 bp, and 98+25 bp, respectively (Figure 2C). Using this restriction fragment length polymorphism (RFLP) assay, we were able to rapidly and accurately identify the genotypes of offspring mice. Due to the loss of *Foxn1*, homozygous NSIN females are poor breeders and fail to lactate. Heterozygous (*Foxn1*$^{+/-}$NSI) females breed well, which were mated with homozygous male NSIN mice to generate the NSIN offspring. The average litter size was about 6 to 8 per litter. Overall, about a half of the mice offspring were NSIN mice. The NSIN mice were athymic and hairless (Figure 2D). Furthermore, B, T and NK cells were absent in NSIN mice, similar to NSI, NOG and NDG mice (Figure 2E). In summary, we generated a novel strain of nude mice by knocking out the *Foxn1* gene by CRISPR/Cas9 from the background of NOD/SCID/IL2rg$^{-/-}$ (NSI).

Loss of *Foxn1* impaired T cell development and thymus regeneration in NSIN mice

Foxn1 is a critical regulator of thymus and T cell development. To compare the capacities of adoptive T cell development between NSI and NSIN mice, we detected the reconstitution of allogeneic bone marrow nucleated cells (BMNCs) (Figure 3A). After 4 weeks, the reconstitution of total donor cells was similar between NSI and NSIN mice (Figure 3B); however, T cell development from BMNCs was markedly impaired in NSIN mice compared to NSI mice (Figure 3C), as indicated by the percentages of donor T cells in the peripheral blood (PB) (Supplemental Figure 1A),

spleen (SP) and bone marrow (BM). Moreover, both CD4 and CD8 T cells were present in NSIN and NSI mice, but the CD4-CD8- compartment increased in NSIN mice compared to NSI mice (Supplemental Figure 1B). Strikingly, 4 weeks after BMNCs transplantation, thymuses were regenerated in NSI mice, but not in NSIN mice (Supplemental Figure 1C), confirming that *Foxn1* is a critical regulator of thymus development. In the regenerated thymus, most thymocytes were derived from the donor mice and most T cells were CD4+CD8+ (Supplemental Figure 1D).

Foxn1 deletion resulted in improved engraftment of leukemic cells in NSIN mice

Immunodeficiency is positively correlated with tumor engraftment capacity[12,34,35]. NSI mice have been widely used in translational studies of human immunology and hematological malignancies[36]. To evaluate the effect of *Foxn1* deletion on the engraftment capacity of hematological cells, we compared the engraftment of a human B cell acute lymphoblastic leukemia (B-ALL) cell line, Nalm6, in NDG, NOG, NSI and NSIN mice. First, Nalm6 cells were labeled with GFP-luciferase using lentiviral transfection followed by FACS sorting on GFP^+ cells (Figure 4A). Subsequently, Nalm6-GL cells were injected into in NDG, NOG, NSI and NSIN mice at low (n=5, for each strain) (Figure 4B), medium (n=5, for each strain) (Figure 4C), and high (n=5, for each strain) (Figure 4D) doses via the tail vein without preconditioning. Upon exhibiting symptoms of paralysis, the mice were sacrificed. The proportions of GFP+ Nalm6-GL cells in PB, SP and BM were higher in NSIN mice than in NDG, NOG and NSI mice (Figure 4B-D, Supplementary Figure 2). To ensure that GFP+ cells were Nalm6-GL cells, we also analyzed human CD19 and found that all GFP+ cells were tumor cells (Figure 4E). Thus, NSIN mice exhibited enhanced capacity of engrafting leukemic cells compared to NDG, NOG and NSI mice.

Foxn1 deletion in NSIN mice resulted in improved engraftment of solid tumors

To evaluate the solid tumor engraftment capacity of NSIN mice, we compared

the engraftment of a human lung adenocarcinoma cell line, A549, in NDG, NOG, NSI and NSIN mice. A549-GL cells were generated similarly to Nalm6-GL cells (Figure 5A). A549-GL cells were subcutaneously injected into NDG, NOG, NSI and NSIN mice at low (n=5, for each strain), medium (n=5, for each strain) and high doses (n=5, for each strain). The A549-GL tumors were significantly larger in NSIN mice than in NDG, NOG and NSI mice, at all three doses (Figure 5B-D). NOG, NSI and NSIN mice bearing subcutaneous A549-GL tumors in the medium and high dose groups were shown (Figure 5E-F) and tumors were most clearly visible in NSIN mice. In contrast to NOG and NSI mice, the blood vessels of the tumors were also clearly visible in NSIN mice.

NSIN mice exhibited higher TEI scores

Previously, we developed a tumor engraftment index (TEI) and a statistical formula [15] for the simple and accurate quantification of the immunodeficiency of mouse strains. The TEI scores for NDG, NOG, NSI and NSIN mice engrafting Nalm6-GL cells were calculated as detailed previously [15] (Figure 6A). The differences between the TEI scores of these three strains were small, indicating significant engrafting of human acute leukemia cells by all of these strains, which is consistent with previous results[12]. However, the TEI score of NSIN mice engrafting A549-GL cells was higher than those of NDG, NOG and NSI strains (Figure 6B). The overall TEI scores for hematological and solid cancers were also calculated, which indicated an overall increase in the immunodeficiency of NSIN mice, compared with NDG, NOG and NSI strains (Figure 6C).

NSIN mice facilitated fluorescence live imaging of xenografts

The hairless phenotype of NSIN mice could be advantageous in tumor watch and imaging. Indeed, another human large cell lung cancer cell line, H460-GL cells, were injected into NSIN and NSI mice, and the subcutaneous tumors showed no difference between NSIN and NSI mice in terms of tumor size and luciferase-derived

bioluminescence; however, tumors in NSIN mice emanated more condensed and sharper GFP fluorescence than NSI mice (Figure 7A). Likewise, the CD123+ human acute myeloid leukemia cell line Molm13-GL were intravenously injected into NSIN and NSI mice, and GFP signals emanating from the spleen and bone marrow of NSIN mice were more distinct than that in NSI mice; moreover, the tumor burden of Molm13-GL cells in NSIN mice were higher than that in NSI mice (Figure 7B). These results indicated that NSIN mice are more suitable hosts for live imaging of autofluorescence derived from xenografts.

Discussion

In basic and translational research, complex biological processes are to be analyzed in vivo, which leads to a demand for new animal models. NSI mice have been used in translational biomedical research in our lab. However, a nude strain with comparable immunodeficiency has not been previously reported. In the present study, we generated nude NSI mice using the CRISPR/Cas9 system on the background of NSI mice by embryonic co-microinjection with Cas9 mRNA and gRNA. The mutant offspring were mated to generate homozygous NSIN mice. These mice were hairless and exhibited more severe immunodeficiency than NSI mice. Hence, NSIN mice provide a new platform for basic and translational research.

Athymic nude mice bearing deletion of *Foxn1* have been widely used in cancer research. *Foxn1* is required to maintain the postnatal thymus, and changes in *Foxn1* expression in TECs may contribute to thymus involution during aging[24]. Our study also revealed that loss of *Foxn1* resulted in blockade of thymus development following allogeneic BMNCs transplantation, which confirmed a critical role of *Foxn1* in thymus function. The postnatal thymus is the primary source of T cells in vertebrates, and thymocyte development requires interactions with TECs. Specifically, *Foxn1* controls the transcription of genes involved in the attraction and lineage commitment of T cell precursors, and regulates the expression of genes involved in antigen processing and thymocyte selection[25]. In consistence, T cell development

from allogeneic BMNCs in NSIN mice was also impaired by deletion of *Foxn1* in our study. Extrathymic development of T cells has been well established [37], which could be responsible for the reduced level, but still presence of T cells in NSIN mice, especially in the spleen.

Nude mice are suitable for research requiring whole-body fluorescence-based imaging techniques[38-40]. The novel NSIN strain derived using the CRISPR/Cas9 system exhibited a hairless phenotype. This was advantageous for observation and imaging of tumors by GFP or other fluorescence. For example, the blood vessels of subcutaneous tumors could be seen through the skin of NSIN mice, making NSIN mice a potentially ideal model for xenograft angiogenesis studies. In addition, NSIN mice exhibited higher immunodeficiency than NSI mice, as indicated by their TEI scores. The elevation of TEI scores caused by *Foxn1* deletion was more significant for subcutaneous solid tumors than for hematological cancers (Figure 5). In our study, NSI mice engrafted tumors more efficiently than NOG mice, which could be attributed to the presence of extracellular domain of IL2rg in NOG mice, but complete IL2rg deletion in NSI mice [3]. *Foxn1* inactivity in nude mice maintains skin in an immature state, resembling neoteny[41-43]. Thus, NSIN mice may also be useful for generating humanized skin models to study human skin regeneration.

In sum, our study generated a new mouse strain and demonstrated the advantages of this strain in xenograft models generation and tumor surveillance.

Materials and methods

Mice

All of the animal experiments were performed in the Laboratory Animal Center of the Guangzhou Institutes of Biomedicine and Health (GIBH). All of the experimental protocols were performed in accordance with the instructional guidelines of the China Council on Animal Care and approved by the Ethics Committee of Animal Experiments at GIBH. C57BL/6J (B6) (RRID: IMSR_JAX:000664), ICR/HaJ (RRID:

IMSR_JAX:009122) and NOG mice were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). Another NOD/SCID/IL2rg$^{-/-}$ strain NDG mice were purchased from Biocytogen (Beijing, China).NSI mice were generated by our group at GIBH[15-19]. NSIN mice were generated using CRISPR-Cas9, as detailed in this paper. All of the mice were bred and maintained in specific pathogen-free (SPF)-grade cages and provided with autoclaved food and water.

DNA constructs

The guide sequence was incorporated into the first exon of the mouse *Foxn1* locus. A pair of oligonucleotides for the targeting site (forward: 5′-caccggaagtgtgacgtcagactg-3′; reverse: 5′-aaaccagtctgacgtcacacttcc-3′) was annealed and ligated into the BbsI site of the gRNA cloning vector (Addgene: 41824). A pcDNA3.3-hCas9 plasmid (Addgene: 41815) was used for in vitro transfection. A pair of oligonucleotides for the targeting site (forward: 5′-ataggaagtgtgacgtcagactg-3′; reverse: 5′-taaaaccagtctgacgtcacacttcc-3′) were annealed and ligated into the BbsI-linearized pUC57-T7 vector (Addgene: 51306), using a T7 promoter for in vitro transcription. The Cas9 expression vector (MLM3613; Addgene: 42251) was used for in vitro transcription. All of the plasmids were provided by Professor Liangxue Lai at GIBH.

Cell culture and transfection

PL08 is an immortalized murine fetal liver cell line, as reported previously[16]. Millicell Hanging Cell Culture Inserts (Millipore, Darmstadt, Germany) were used to culture PL08 cells. PL08 cells (1×10^6) were transfected with U6-sgRNA vectors (10 μg) and pcDNA3.3-hCas9 plasmids (30 μg). Cells were selected using G418 (500 μg/ml; Sigma) after 24 h of transduction, and were maintained in selection medium for 72 h. Single clones were collected and seeded in each well of a 96-well plate. Monoclonal cells were expanded. Then, DNA of monoclonal cells was extracted to determine their genotypes using DNA sequencing. Nalm6, Molm13, A549 and H460 cells were cultured in RPMI-1640 media (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Biochrom, Australia) and 1% penicillin/streptomycin. HEK-293T cells

were used for lentivirus production and cultured in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. Lentivirus particles were produced in HEK-293T cells via polyethyleneimine (Sigma-Aldrich, St Louis, MO, USA) transfection. A pWPXLd-based (luciferase-2A-GFP) lentiviral plasmid and two packaging plasmids, psPAX2 and pMD.2G, were co-transduced into HEK-293T cells. The supernatant containing luciferase-2A-GFP was filtered through a 0.45-μm filter, and then used to transfect the Nalm6, Molm13, A549 and H460 cells. The Nalm6-GL (GFP-luciferase), Molm13-GL, A549-GL and H460-GL cells were sorted using FACSAria™ II (Becton Dickinson, San Jose, CA, USA) for culture.

Transcription in vitro

The Cas9 expression vector (MLM3613) was linearized with Pme I (Thermo Fisher Scientific, Waltham, MA, USA) and used as the template for in vitro transcription (IVT) using an mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Ambion, AM1345). Cas9 mRNA was purified using an RNeasy Mini Kit (Qiagen, 74104). The gRNA templates for in vitro transcription of RNA were purified PCR products obtained from pUC57-T7-*Foxn1*-gRNA vectors using a primer pair (T7-F: 5′-gaaattaatacgactcactata-3′ and T7-R: 5′-aaaaccgactcggtgccacaaaagc-3′) and a high-fidelity enzyme (Takara). The T7-sgRNA PCR product was gel purified and used as the template for IVT with a MEGAshortscript T7 Transcription Kit (Ambion, AM1354). The gRNA was purified using a MEGAclear Kit (Ambion, AM1908) and concentrated by alcohol precipitation.

Microinjection of single-cell embryos

NSI and ICR/HaJ mouse strains were used as zygote donors and foster mothers, respectively. Female NSI mice (aged 8–10 w) were superovulated using intraperitoneal injections of pregnant mare serum gonadotropin (5 IU; Sigma) and human chorionic gonadotropin (5 IU; Sigma) at 48-h intervals. The superovulated female mice were mated to NSI male mice, and the fertilized embryos were collected from the oviducts. Cas9 mRNA (20 ng/μl) and sgRNA (20 ng/μl) were injected into the cytoplasm of fertilized eggs with visible pronuclei in M2 medium (Sigma) using a

piezo-driven micromanipulator. The injected zygotes were cultured in KSOM with amino acids at 37℃ under 5% CO_2 in air until the blastocyst stage (3.5 d). The surviving embryos were selected and transferred into the uterus of pseudo-pregnant foster mothers. The resulting offspring were analyzed for edited *Foxn1* genes.

DNA extraction and genotyping

Monoclonal cells and genomic DNA from mouse tail samples were extracted using a Universal Genomic DNA Extraction Kit Ver.3.0 (DV811A, Takara, Japan) according to the manufacturer's instructions. Genomic DNA was subjected to PCR amplification, and mutations were identified by direct sequencing. For sequencing, the primer pair *Foxn1*-F1 (5′-aatttctcaccttggctatc-3′), *Foxn1*-R1 (5′-caggagtcccaaagtgacgg-3′) was used to amplify 644 bp PCR products spanning the target site. After identifying the mutation site, the primer pair *Foxn1*-F2 (5′-ttcgaggccaggactgggtg-3′), *Foxn1*-R2 (5′-ttacgttctgtggggcaggg-3′) was used to amplify 123 bp PCR products spanning the mutation site. The PCR products were digested with the Alu I enzyme (Fermentas; Thermo Fisher Scientific).

FACS analysis

Cells isolated from PB, BM and SP of mice were subjected to flow cytometric analyses. The cells were labeled with FACS antibodies (anti-mCD19-PE, anti-mCD3-APC, anti-mNKp46-PE, anti-mCD3-FITC, anti-mCD4-PE, anti-mCD8-Percp-cy5.5, anti-mCD45.1-PE, anti-mCD45.2-APC) on ice for 20 min. All of the antibodies were obtained from eBioscience (San Diego, CA, USA).Nalm6-GL cells were labeled with anti-hCD19-APC and Molm13-GL cells were labeled with anti-hCD123-APC. Flow cytometric analyses were performed using FACSAria™II. All of the data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Bone marrow transplantation

Bone marrow–nucleated cells (BMNCs; 4×10^6) from C57BL/6J (B6) donor mice (CD45.2+) were injected into the retro-orbital venous sinus of lethally irradiated (2 Gy) NSI (CD45.1+) and NSIN (CD45.1+) mice, respectively. The engraftment of

CD45.2+ donor cells were detected every week in the PB of recipients. 4 weeks later, five animals in each group were killed after bone marrow transplantation (BMT) to analyze the repopulation efficiency of donor cells and the development of donor T cells in the recipients.

Engraftments of leukemia and solid tumors

To directly compare the efficiency of cancer cell engraftment, 1×10^4 (L), 1×10^5 (M), or 1×10^6 (H) NALM6-GL cells suspended in phosphate buffer solution (PB; 0.2 mL) were injected into the tail veins of NOG, NSI and NSIN mice. Similarly, 1×10^4 (L), 1×10^5 (M), or 1×10^6 (H) A549-GL cells suspended in PBS (0.2 mL) were injected subcutaneously into NOG, NSI and NSIN mice. The end points were based on animal models in widespread use. The engraftment of leukemia was measured by analyzing the GFP^+ cells in the PB weekly, or when the mice were moribund after grafting.

Fluorescence live imaging

In vivo whole-body imaging of GFP-labeled cells was performed using a cooled CCD camera system (IVIS 100 Series Imaging System, Xenogen, Alameda, CA, USA). Before in vivo imaging, mice burdened with H460-GL cells or Molm13-GL cells were anaesthetized with isofurane. Quantification of total and average emissions was performed using Living Image software (Xenogen).

Quantification of mouse immunodeficiencies (TEI)

The hematologic TEI was calculated using the following equation: $I_{\text{strain}-\text{hematologic tumor}-n}$= Gi/Di, where “strain” is the immunodeficient mouse strain, “hematologic tumor” is the type of tumor cells, *n* is the number of individuals, “Gi” is the sum of the percentages of tumor cells in the peripheral blood (GPB), bone marrow (GBM), and spleen (GSP) of an individual mouse either during morbidity or after death, and “Di” is the lifespan of the individual mouse after injection of the tumor cells. The solid TEI was calculated using the following equation: $I_{\text{strain}-\text{solid tumor}-n}$= Wi/D, where “strain” is the immunodeficient mouse strain, “solid tumor” is the type of transplanted tumor cells, *n* is the number of individuals, “Wi” is the weight of the graft in an individual mouse either during morbidity or after death, and “D” is the survival time

of the mouse after injection of the tumor cells. The total TEI score was determined as follows: $I_{strain-xenograft-n} = (I_{strain-Nalm6-n} + I_{strain-A549-n})/2$. Calculations were performed using tools on the website of In Vivo Biomedical (http://www.nsitei.com)[15].

Statistical analyses

Data are presented as means ± standard deviation. Student's *t*-test was used to determine the statistical significance of differences between samples. $P < 0.05$ was considered to be significant. All statistical analyses were performed using Prism software, version 5.0 (GraphPad, Inc., San Diego, CA, USA).

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Author Contributions

XW contributed to the conception and design, collection and/or assembly of data, data

analysis and interpretation, and manuscript writing. YL, BL, LQ, YX and QL contributed to the provision of study material, collection and/or assembly of data. BL, SL, SW, and QW provided animal care and administrative supports. YY and DP contributed to the conception and design and provided financial support. GH, QD, PL, DW, and LL contributed to the conception and design. XW and PL contributed to the conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript and provided financial support. All authors read and approved the final manuscript.

Conflict of Interest Statement

The authors declare no competing financial interests.

Figure legends

Figure 1.*Foxn1* gene targeting in PL08 cells using a type II CRISPR system in vitro. (A) Schematic of the Cas9/sgRNA-targeting site in exon 1 of*Foxn1*. The sgRNA-targeting and protospacer-adjacent motif (PAM) sequences are labeled in red and blue, respectively. (B) Schematic of *Foxn1*gene targeting in PL08 cells in vitro. PL08 cells were transfected with U6-*Foxn1*-sgRNA vector and pcDNA3.3-hCas9 plasmid. Abbreviations: U6 = U6 polymerase III promoter; CMV = cytomegalovirus promoter; NLS = nuclear localization signal; TK = thymidine kinase; pA = polyadenylation signal. In the plate wells, living cells are shown in blue and dying cells are shown in gray. (C) Genotyping of *Foxn1*-targeted and wild type (WT) PL08 cells. Clones 1 and 9 are shown. The target exon 1 of *Foxn1* was amplified by PCR and then analyzed using DNA sequencing. Double sequencing peaks in clone 1 showed mutant alleles. The three sequencing peaks in clone 9 may originate from a

mixture of two different clones. (D) Efficient knockout of the *Foxn1* gene in PL08 cells by co-transfection with U6-*Foxn1*-sgRNA vectors and pcDNA3.3-hCas9 plasmids. The percentages indicate *Foxn1*-knockout clones in different groups. Data were representative of three independent experiments. 20 clones were picked and analyzed in each experiment.

Figure2. Generation and characterization of *Foxn1* deleted NSIN mice. (A) Schematic of the generation of NSIN mice. Cas9 mRNA and gRNA were co-injected into the cytoplasm of the pronuclear-stage mouse embryos. The injected embryos were then transferred into pseudopregnant surrogate mothers. The mouse pups were genotyped and screened for *Foxn1* mutations. The in vitro transcription of both vectors was controlled by T7 promoters. (B) Sequence analysis of the founder mice. The target exon 1 of *Foxn1* was amplified using PCR and then analyzed by DNA sequencing. The PCR products were 644 bp long. The WT sequence of *Foxn1* was shown at the top of the figure. Double sequencing peaks indicated the presence of mutant alleles in the somatic cells of the mice. The targeting and PAM sequences are labeled in red and blue, respectively. A new AluI enzyme locus was generated by deleting a thymidine. (C) PCR genotyping of the F2 generation offspring. Primers were designed to amplify 123 bp PCR products spanning the mutation site. The PCR products were digested using the Alu I enzyme. Results from seven offspring mice were shown: WT NSI (lane2 and 5), *Foxn1*$^{+/-}$NSI (lane 1, 6 and 7), and *Foxn1*$^{-/-}$NSI (NSIN) mice (lane 3 and 4). (D) Photographs of thymus of ICR and NSIN mice. (E) Flow cytometric analysis indicating the absence of B, T and NK cells in the peripheral blood (PB) of B6, NOG, NSI, NSIN and NDG mice. Cells were gated from FSC/SSC for the detection of T, B and NK cells. B6 mice were used as a positive control.

Figure 3. Allogeneic T cell development was affected in NSIN mice. (A) Schematic of the bone marrow transplantation (BMT). Bone marrow–nucleated cells (BMNCs; 4×10^6) from C57BL/6J (B6) donor mice (CD45.2+) were injected into the retro-orbital venous sinus of lethally irradiated (2 Gy) NSI (CD45.1+) and NSIN (CD45.1+) mice, respectively. The engraftment of CD45.2+ donor cells were detected every week in

the PB of recipients. 4 weeks later, five animals in each group were killed BMT to analyze the repopulation efficiency of donor cells and the development of donor T cells in the recipients. (B) At 4 weeks, BMNCs from B6 mice reconstituted in both NSI and NSIN mice at similar levels in PB. CD45.2+ cells were derived from B6 mice.(C)The repopulation percentage of CD45.2+ donor cells (left) and CD45.2+CD3+ T cells (right) in PB,SP and BM. n=5 for each group. Error bars denote standard errors of the means (SEM). Groups were compared using the two-tailed unpaired *t*-test. * $P< 0.05$. ** $P < 0.01$. *** $P< 0.001$.

Figure 4. Engraftment efficiencies of Nalm6-GL cells in NDG, NOG, NSI, and NSIN mice. (A) Establishment of Nalm6-GL cell line. Nalm6 cells were transfected with luciferase-GFP-expressing lentiviral vectors, and sorted on GFP+ cells. Nalm6-GL cells were labeled human CD19+ and GFP+. (B–D) Percentages of Nalm6-GL cells in PB, SP, and BM of NDG, NOG, NSI, and NSIN mice injected with (B) low (1×10^4, n= 5 for each strain), (C) medium (1×10^5, n= 5 for each strain), and (D) high numbers (1×10^6, n= 5 for each strain) of Nalm6-GL cells. Nalm6-GL cells were injected into each mouse through the tail vein, and analyzed on day 20, 24, or 32 for the low, medium, and high dose groups, respectively. (E) Engraftment efficiencies of Nalm6-GL cells were further confirmed by labeling with human CD19+ in the BM from the high dose (1×10^6) group. Error bars denote standard errors of the means (SEM). Groups were compared using the two-tailed unpaired *t*-test. * $P< 0.05$. ** $P < 0.01$. *** $P< 0.001$.

Figure 5. Engraftment efficiencies of A549-GL cells in NDG, NOG, NSI, and NSIN mice. (A) Establishment of A549-GL cell line. Lung adenocarcinoma A549 cells were transfected with luciferase-GFP-expressing lentiviral vectors, and sorted on GFP^+ cells. (B–D) Weight of A549 tumors in NDG, NOG, NSI and NSIN mice subcutaneously injected with (B) low (1×10^4, n= 5 for each strain), (C) medium (1×10^5, n=5 for each strain), and (D) high numbers (1×10^6, n=5 for each strain) of A549-GL cells and analyzed on day 30. (E–F) Photographs of NOG, NSI, and NSIN micebearing subcutaneous A549-GL tumors from the (E) medium (n=5 for each strain)

and (F) high groups (n=5 for each strain) on day 30. The mice in the low group bore no visible tumors and are not shown. Error bars denote SEM. Groups were compared using the two-tailed unpaired *t*-test. * P < 0.05. ** P < 0.01. *** P < 0.001.

Figure 6. NSIN mice showed higher tumor engraftment index (TEI) scores than NDG, NOG, NSI mice. (A) TEI scores of NDG, NOG, NSI and NSIN micefor Nalm6 cells. (B) TEI scores of NDG, NOG, NSI and NSIN mice for A549 cells. (C) Overall TEI scores of NDG, NOG, NSI and NSIN mice for both leukemia and solid tumors.Xenograft TEI scores as indicators of immunodeficiency.

Figure7. Improved fluorescence live imaging in NSIN mice. (A) Fluorescence live imaging of H460-GL burdened mice. 1×10^6 H460-GL cells were subcutaneously injected into NSIN and NSI mice (n=3 for each group) and luciferase and GFP were analyzed on day 30. Total flux of luciferase-derived and GFP-derived fluorescence was compared between NSI and NSIN mice (right panel). H460-GL, a human large cell lung cancer cell line, expressed GFP and luciferase (GL). (B) Fluorescence live imaging of Molm13-GL burdened mice. 1×10^6 Molm13-GL cells were injected into NSIN and NSI mice (n=4 for each group) through the tail vein and GFP fluorescence was analyzed on day 30. Left,the AML cell line Molm13-GL expressed CD123 and GFP; middle, fluorescence live imaging of Molm13-GL cells; right, comparison of the total flux of GFP fluorescence between NSI and NSIN mice. Error bars denote SEM. Groups were compared using the two-tailed unpaired *t*-test. * P < 0.05. ** P < 0.01. *** P < 0.001.

Supplementary Figure 1. NSIN mice are more resistant to allogeneic T cell development than NSI mice. (A) Donor BMNCs reconstitution in the T (CD45.2+ CD3+) cell lineage in PB of NSIN (upper) and NSI (lower) mice (n=5 for each group) 4 weeks after bone marrow transplantation (BMT). (B) Donor T cells were analyzed for CD4 and CD8 expression by flow cytometry. (C) Loss of Foxn1 inhibited the regeneration of thymuses after BMT in NSIN mice. (D) Phenotype of cells from the regenerated thymuses in NSI mice.

Supplementary Figure 2. Representative FACS analysis of Nalm6-GL cells in NOG, NSI, and NSIN mice. (A–C) Percentages of Nalm6-GL cells in PB, SP, and BM of mice intravenously injected with (A) low (1×10^4), (B) medium (1×10^5), and(C) high (1×10^6) numbers of Nalm6-GL cells. GFP^+ cells represent Nalm6-GL cells.

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